PLATELET ADHESION AND PLASME-PROTEIN ADSORPTION ON POLYACRYLONITRILE CONTAINING POLY(ETHYLENE OXIDE) SIDE CHAINS

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Polyacrylonitrile graft copolymers having poly(ethylene oxide) side chains of different number of ethylene oxide unit were synthesized. For the copolymers, adhesion of platelets was examined by a column method, whereby a platelet suspension was passed through a column of glass beads precoated with the copolymer. Independently, adsorption of rat serum albumin (RSA), γ-globulin (RγG) and fibrinogen (RPF) to the polymer surface was examined in a batch system.

Thus, it was found that the degree of the protein adsorption on the polymer surface was in the order of RPF > RγG > RSA and reduction of the platelet retention for the protein-coated polymer was also in the same order as that of the protein adsorption. From the results, it was concluded that the protein adsorbed on the polymer surface prevents the platelet retention.

Key words: Nonthrombogenic polyacrylonitrile/Platelet adhesion/Poly(ethylene oxide) side chain/Adsorption of plasma proteins/Adsorption of fibrinogen

1. Introduction

Blood compatibility of graft copolymers having poly(ethylene oxide) (PEO) side chains has been studied by many researchers. Mori et al.[1] synthesized poly(vinyl chloride) graft copolymers with PEO side chains and showed that the copolymer had excellent nonthrombogenicity, degree of which was higher for the copolymer having longer PEO side chains. Thus, they concluded that the volume restriction effect resulting from the formation of longchain PEO on the surface effectively suppresses the adsorption of blood elements and prevents the denaturation of blood elements. They also observed that double layer of the plasma proteins between the polymer surface and blood and suggested that the double layer plays a role as buffer to minimize denaturation of blood elements and that the buffer action of the double layer is caused by superior flexibility, hydrophilicity and blood compatibility of the long chain PEO. Thereafter, Nagaoka et al.[2] proposed another explanation that the motion of the PEO side chains with water molecule bound to the PEO units with water molecule bound to the PEO units causes microscopic water flow which prevents the local stagnation of blood elements and the adhesion of platelets. Han et al.[3] supported the explanation of Nagaoka et al. from the results on the blood compatibility of PEO grafted polyurethane.

Although it is well known[4] that adsorbed protein layer plays an important role in the blood compatibility of the polymer surface, not much known about the adsorption of proteins on the polymer surface. Recently, Miyama et al.[5] found that adsorption of serum proteins prevents the retention of platelets on surface of copolymers having poly(ethylene oxide) and dimethylamino side chains. In order to confirm the role of adsorbed proteins, we synthesized polyacrylonitrile graft copolymers having PEO side chains of different lengths and examined platelet adhesion and plasma protein adsorption on the graft copolymer surface.

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2. Methods

2.1 Preparation of Graft Polymer

The preparation of the graft polymer was performed according to the method described previously[5,6] and will be described here briefly. The trunk polymer was synthesized by the photopolymerization of acrylonitrile with carbon tetrabromide as an initiator in dimethylsulfoxide (DMSO). The obtained polymer was photosensitive and contained bromine atoms and will be abbreviated as "PANBr". At various feeding ratios, PANBr and methoxypolyethyleneglycol methacrylate (MnG ; prepared by Shin-Nakamura Chemical Co., Ltd.),

$$CH_2 = C(-CH_3)-C(=O)-(OCH_2CH_2)n-OCH_3 \quad ,$$

were dissolved into DMSO. Here n is 4, 9, 23 or 110. The mixture was photoirradiated from the outside of the vessel at 25° C for 6 h in nitrogen flow with a 100-W high-pressure mercury lamp. The reacted solution was concentrated at reduced pressure and poured into a large quantity of aqueous methyl alcohol. The precipitated polymer was thoroughly washed with distilled water and methylalcohol at 50°C and dried at 40°C. The polymer was purified by double precipitations.

2.2 Evaluation of Polymer Properties.

The molecular weight of PANBr was measured by gel permeation chromatography (Waters, GPC-224) using an N,N-dimethylformamide (DMF) solution, with calibration using polystyrene standards. The composition of the copolymer was determined by ^{1}H-NMR spectroscopy (JEOL, JNM-GX270), where spectra were measured by using 10 mg of each polymer dissolved into 0.5 ml DMSO-d_6 in an NMR tube. The water content of the copolymer was determined from the difference between the weight of the copolymer soaked in distilled water for more than 48 h and that dried in a vacuum at 60° C for 10 h, expressed by the ratio(%) of the water content to the weight of wet film.

2.3 Measurement of protein adsorption

Two g of glass beads (150-250 mesh) precoated with the polymer were immersed into 10 mL HBSS-HEPES, containing 0.2 mg rat serum albumin (RSA, Sigma Fraction V), rat γ-globulin (RγG, Sigma Fraction II, IV) or rat plasma fibrinogen (RPF, Sigma Fraction I) and incubated at pH7.3 for 6 h at 30°C. Here, HBSS-HEPES was prepared by adding N-2-hydroxyl-N'-2-sulfoethylpiperazine (HERPES) to Hank's balanced salt solution (HBSS). Concentration of the proteins were measured before and after the incubation by adding Coomassie Brilliant Blue G-250 to the solution and measuring absorption at 595 nm. The amount of the proteins adsorbed was calculated from the difference in the protein concentration before and after the incubation.

2.4 Measurement of platelet adhesion

Details of the method were reported previously[7]. The polymer was coated on glass beads (48-60 mesh, Toshiba Ballotini Co.). Then, 1 g of precoated beads was closely packed in a polyvinylchloride tube (10 cm length and 3 mm internal diameter).

About 10 ml of blood was withdrawn by cardiopuncture from a Wistar male rat into syringe with 1 mol of 0.25% sodium citrate saline solution. Platelets rich plasma (PRP) was separated by centrifugal operation for 20 min at 900 rpm. From the PRP, platelets were precipitated by centrifugal operation for 15 min at 2000 rpm. The platelets were suspended in HBSS-HEPES. The platelet concentration in the suspension was adjusted to ca. 1×10^7 cell/ml.

The platelet suspension from a disposable syringe was passed through the column with the use of Precidol Model 5003 infusion pump for 7 min at a flow rate of 0.2 ml/min unless otherwise described. The column had been primed with HBSS to exclude a liquid-air interface. The number of platelets in the solution before and after the elution was determined by a Coulter counter (Coulter Electronic Inc.). Retention (percentage) of the platelets was calculated according to the following equation :

$$(1-[P]/[P]_0)\times100$$

where $[P]_0$ and $[P]$ are concentrations of the platelets before and after the elution. The retention value is an average of four runs. The variations of

the data were much less than those for protein absorption.

Here, precoating the polymer-coated beads with proteins was performed by introducing protein solution into the column packed with the polymer-coated beads in closed system for one night and by rinsing the buffer solution before the measurement. Also, precoating the polymer-coated beads with PPP was made by incubating the packed polymer-coated beads in buffer solution of PPP for 30 min in the column. Instead of the platelet suspension prepared as described above, platelet suspension in PPP prepared by suspending platelets in PPP diluted with the buffer solution was used sometimes for the platelet adhesion measurement.

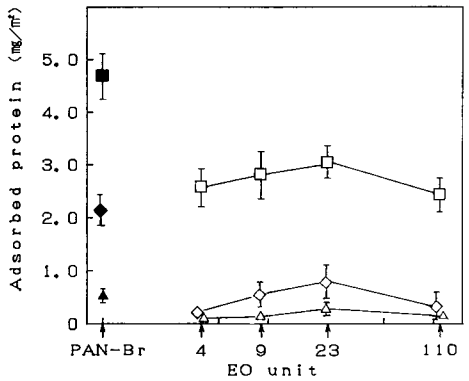

Fig. 1 Equilibrated amount of plasma proteins, RSA (△, ▲), RγG (◇, ◆), and RPF (□, ■), adsorbed to the surfaces of graft copolymers of different PEO lengths and for trunk polymer (PAN-Br).

3. Results and discussion

Composition and water content of the graft copolymers are shown in Table 1, where the average molecular weight of the PANBr used for the photograftpolymerization was 8.2×10^5 and water content of all samples was kept the same.

In Fig. 1, equilibrated amount of RSA, RγG and RPF adsorbed on the polymer surface for the graft-copolymers are shown, where data for the PANBr are shown for comparison and vertical lines indicate standard variation. The amount of protein adsorbed shows a maximum at n = 23 for the polymers examined.

Platelet retention for the graftpolymers when the polymer-coated beads are precoated with RSA and RPF by using 0.005 weight % protein solution are shown in Fig. 2 together with that for the uncoated polymer, where data for the PANBr are shown for comparison. The platelet retention shows a maximum at n = 23 both for the uncoated polymer and RSA-coated polymer, but shows almost no dependence on the number of EO unit for RPF-coated polymer. Platelet retention when protein solution of much higher concentration (0.1 weight %) is used for protein coating are shown in Fig. 2. The platelet retention shows no dependence on the number of EO unit both for RSA- and RγG-coated polymers.

From Fig. 1, the degree of the protein adsorption of the polymer surface is found to be in the order of RPF > RγG > RSA. On the other hand, Fig. 2 shows that reduction of the platelet retention for the protein-coated polymer is in the order of RPF > RγG > RSA. The results support the conclusion of

Table 1. Composition and water content of polymers.

PEO length n	Composition MnG (wt%)	MnG (m mol/g)	EO (m mol/g)	Water content (%)
4	39.7	1.438	5.75	32
9	37.0	0.747	6.73	32
23	36.9	0.332	7.63	34
110	29.2	0.059	6.48	31

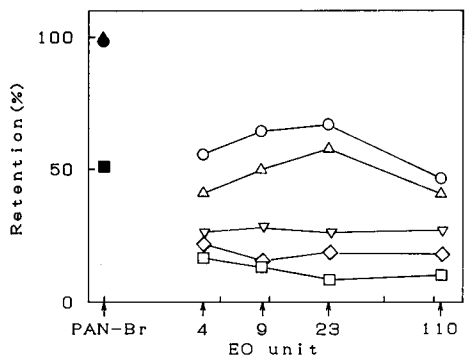

Fig. 2 Platelet retention on the surface of graft copolymers of different PEO lengths in platelet suspension. Uncoated ○, ●: Coated by treating with 0.005 wt% (△, ▲) and 0.1 wt% (▽) of RSA, 0.005 wt% RPF (□, ■), and 0.1 wt% RγG (◇) solutions.

Miyama at al.[5] that the protein adsorbed to the PEO-grafted surface is essential for the elimination of platelet adhesion.

In Fig. 3, effect of Ca^{2+} addition on the platelet retention for RPF-coated polymer is shown, where the addition of Ca^{2+} increases slightly the platelet adhesion. It is known[8] that the presence of Ca^{2+} enhances fibrinogen binding to activated platelets via GPII b/IIIa complexes. Therefore, it is expected that the addition of Ca^{2+} increases markedly the platelet adhesion. The obtained affect is too small to conclude that the adhered platelets are activated by the Ca^{2+} addition.

Figure 4 shows platelet retention for the graft copolymers when the polymer-coated beads are precoated with PPP by using buffer solution containing 0.5 weight %, 0.25 weight % and 0.125 weight % PPP, respectively, where data for the PANBr are shown for comparison. It is obvious that the adsorbed PPP reduces markedly the platelet retention and that the degree of the reduction is higher for the polymer coated by the buffer solution of higher PPP concentration.

In all of the platelet retention measurements described above, buffer solution of platelet suspension was used. In the measurement shown in Fig. 5, flow of buffer solution containing 2.5 weight %, 0.5 weight %, 0.25 weight % and 0.125 weight % PPP are used respectively, where the polymer-coated beads are coated with PPP by using buffer solution

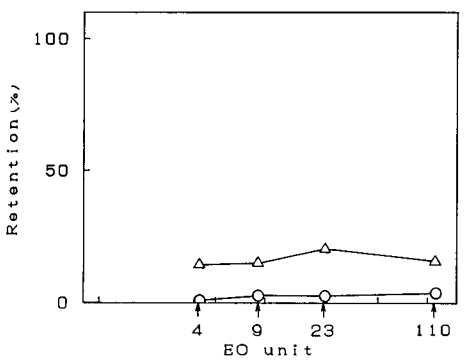

Fig. 3 Platelet retention on the RPF-coated surface of graft copolymers of different PEO lengths in platelet suspension, in the presence (●) and absence (○) of Ca^{2+} ions.

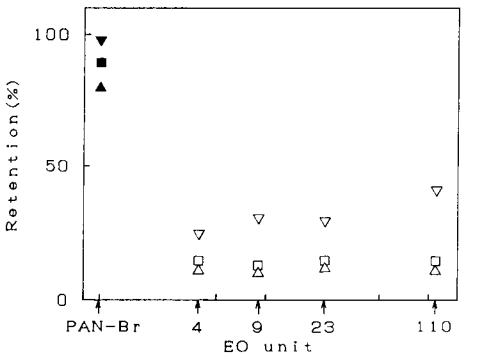

Fig. 4 Platelet retention on the surface of graft copolymers of different PEO lengths in platelet suspension. Pretreated with PPP solution of 0.5 wt% (△, ▲), 0.25 wt% (□, ■), and 0.125 wt% (▽, ▼).

containing the corresponding concentration of PPP. The figure shows that reduction of platelet retention is higher for the buffer solution of higher PPP concentration but that the difference is not large.

The results shown in Figs. 4 and 5 indicate that the plasma proteins adsorbed to the polymer surface prevent the retention of platelets and that the degree of the grevention does not depend upon the PEO length.

Why the protein adsorption in Fig. 1 and the platelet adhesion in Fig. 2 show a maximum at n = 23 cannot be explained at present. However, both the protein adsorption and the platelet adhesion seems to proceed *via* the same mechanism in the

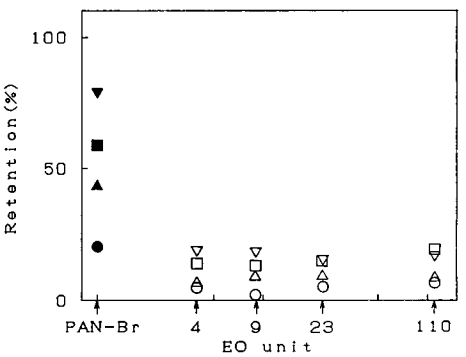

Fig. 5 Platelet retention on the surface of graft copolymers of different PEO lengths in 2.5 wt% (○, ●), 0.5 wt% (△, ▲), 0.25 wt% (□, ■), and 0.125 wt% (▽, ▼) PPP solutions. The surface was pretreated with PPP solution of corresponding concentration.

present condition, perhaps *via* physico-chemical adsorption. This is supported by the Ca^{2+} addition experiment. The present results are inconsistent with the conclusion of Nagaoka et al.[2] that the motion of the PEO side chains with water molecules bound to the PEO units causes microscopic water flow, which prevents the local stagnation of blood components and the adhesion of platelets and that the effect is more marked for the polymer having longer PEO side chains. Rather the present results are consistent with findings of Mori et al.[1] that the adsorbed proteins penetrate into the PEO layer to form a microheterogeneous PEO/protein layer and that this proteinaceous layer prevents the adhesion of the platelets. At any rate, contact time between the polymer and platelets is limited to several to 30 minutes in the present experimental condition. Therefore, the present experimental results are limited to the initial reversible or physico-chemical adsorption of platelets. The competitive adsorption and desorption of plasma proteins and the activation and deformation of the adsorbed proteins must be studied to confirm the dependence of platelet adhesion on the PEO length observed in the *in vivo* test[1,3].

4. Conclusion

Degree of the adsorption of plasma proteins on the PEO-grafted polyacrylonitrile was in the order of RPF > RγG > RSA and the retention of platelet on the protein-coated polymers was in the reverse order as that of the protein adsorption. The results support the conclusion that the plasma protein adsorbed on the PEO-grafted polymer surface prevents the platelet retention.

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